

1           BIOPOLYMER MARKER INDICATIVE OF DISEASE STATE HAVING  
2                   A MOLECULAR WEIGHT OF 1998 DALTONS  
3

4   FIELD OF THE INVENTION

5           This invention relates to the field of characterizing the  
6   existence of a disease state; particularly to the utilization  
7   of mass spectroscopy to elucidate particular biopolymer markers  
8   indicative of disease state, and most particularly to specific  
9   biopolymer sequences having a unique relationship to at least  
10   one particular disease state.  
11

12   BACKGROUND OF THE INVENTION

13           Methods utilizing mass spectrometry for the analysis of a  
14   target polypeptide have been taught wherein the polypeptide is  
15   first solubilized in an appropriate solution or reagent system.  
16   The type of solution or reagent system, e.g., comprising an  
17   organic or inorganic solvent, will depend on the properties of  
18   the polypeptide and the type of mass spectrometry performed and  
19   are well known in the art (see, e.g., Vorm et al. (1994) Anal.  
20   Chem. 66:3281 (for MALDI) and Valaskovic et al. (1995) Anal.  
21   Chem. 67:3802 (for ESI). Mass spectrometry of peptides is  
22   further disclosed, e.g., in WO 93/24834 by Chait et al.

23           In one prior art embodiment, the solvent is chosen so that

1 the risk that the molecules may be decomposed by the energy  
2 introduced for the vaporization process is considerably  
3 reduced, or even fully excluded. This can be achieved by  
4 embedding the sample in a matrix, which can be an organic  
5 compound, e.g., sugar, in particular pentose or hexose, but  
6 also polysaccharides such as cellulose. These compounds are  
7 decomposed thermolytically into CO<sub>2</sub> and H<sub>2</sub>O so that no residues  
8 are formed which might lead to chemical reactions. The matrix  
9 can also be an inorganic compound, e.g., nitrate of ammonium  
10 which is decomposed practically without leaving any residues.  
11 Use of these and other solvents are further disclosed in U.S.  
12 Pat. No. 5,062,935 by Schlag et al.

13 Prior art mass spectrometer formats for use in analyzing  
14 the translation products include ionization (I) techniques,  
15 including but not limited to matrix assisted laser desorption  
16 (MALDI), continuous or pulsed electrospray (ESI) and related  
17 methods (e.g., IONSPRAY or THERMOSPRAY), or massive cluster  
18 impact (MCI); these ion sources can be matched with detection  
19 formats including linear or non-linear reflection time-of-  
20 flight (TOF), single or multiple quadropole, single or multiple  
21 magnetic sector, Fourier Transform ion cyclotron resonance  
22 (FTICR), ion trap, and combinations thereof (e.g., ion-  
23 trap/time-of-flight). For ionization, numerous

1 matrix/wavelength combinations (MALDI) or solvent combinations  
2 (ESI) can be employed. Subattomole levels of protein have been  
3 detected, for example, using ESI (Valaskovic, G. A. et al.,  
4 (1996) Science 273:1199-1202) or MALDI (Li, L. et al., (1996)  
5 J. Am. Chem. Soc. 118:1662-1663) mass spectrometry.

6 ES mass spectrometry has been introduced by Fenn et al.  
7 (J. Phys. Chem. 88, 4451-59 (1984); PCT Application No. WO  
8 90/14148) and current applications are summarized in recent  
9 review articles (R. D. Smith et al., Anal. Chem. 62, 882-89  
10 (1990) and B. Ardrey, Electrospray Mass Spectrometry,  
11 Spectroscopy Europe, 4, 10-18 (1992)). MALDI-TOF mass  
12 spectrometry has been introduced by Hillenkamp et al. ("Matrix  
13 Assisted UV-Laser Desorption/Ionization: A New Approach to Mass  
14 Spectrometry of Large Biomolecules," Biological Mass  
15 Spectrometry (Burlingame and McCloskey, editors), Elsevier  
16 Science Publishers, Amsterdam, pp. 49-60, 1990). With ESI, the  
17 determination of molecular weights in femtomole amounts of  
18 sample is very accurate due to the presence of multiple ion  
19 peaks which all could be used for the mass calculation.

20 The mass of the target polypeptide determined by mass  
21 spectrometry is then compared to the mass of a reference  
22 polypeptide of known identity. In one embodiment, the target  
23 polypeptide is a polypeptide containing a number of repeated

1 amino acids directly correlated to the number of trinucleotide  
2 repeats transcribed/translated from DNA; from its mass alone  
3 the number of repeated trinucleotide repeats in the original  
4 DNA which coded it, may be deduced.

5 U.S. Patent No. 6,020,208 utilizes a general category of  
6 probe elements (i.e., sample presenting means) with Surfaces  
7 Enhanced for Laser Desorption/Ionization (SELDI), within which  
8 there are three (3) separate subcategories. The SELDI process  
9 is directed toward a sample presenting means (i.e., probe  
10 element surface) with surface-associated (or surface-bound)  
11 molecules to promote the attachment (tethering or anchoring)  
12 and subsequent detachment of tethered analyte molecules in a  
13 light-dependent manner, wherein the said surface molecule(s)  
14 are selected from the group consisting of photoactive  
15 (photolabile) molecules that participate in the binding  
16 (docking, tethering, or crosslinking) of the analyte molecules  
17 to the sample presenting means (by covalent attachment  
18 mechanisms or otherwise).

19 PCT/EP/04396 teaches a process for determining the status  
20 of an organism by peptide measurement. The reference teaches  
21 the measurement of peptides in a sample of the organism which  
22 contains both high and low molecular weight peptides and acts  
23 as an indicator of the organism's status. The reference

1 concentrates on the measurement of low molecular weight  
2 peptides, i.e. below 30,000 Daltons, whose distribution serves  
3 as a representative cross-section of defined controls.  
4 Contrary to the methodology of the instant invention, the '396  
5 patent strives to determine the status of a healthy organism,  
6 i.e. a "normal" and then use this as a reference to  
7 differentiate disease states. The present inventors do not  
8 attempt to develop a reference "normal", but rather strive to  
9 specify particular markers which are evidentiary of at least  
10 one specific disease state, whereby the presence of said marker  
11 serves as a positive indicator of disease. This leads to a  
12 simple method of analysis which can easily be performed by an  
13 untrained individual, since there is a positive correlation of  
14 data. On the contrary, the '396 patent requires a complicated  
15 analysis by a highly trained individual to determine disease  
16 state versus the perception of non-disease or normal  
17 physiology.

18 Richter et al, Journal of Chromatography B, 726(1999) 25-  
19 35, refer to a database established from human hemofiltrate  
20 comprised of a mass database and a sequence database. The goal  
21 of Richter et al was to analyze the composition of the peptide  
22 fraction in human blood. Using MALDI-TOF, over 20,000  
23 molecular masses were detected representing an estimated 5,000

1 different peptides. The conclusion of the study was that the  
2 hemofiltrate (HF) represented the peptide composition of  
3 plasma. No correlation of peptides with relation to normal  
4 and/or disease states is made.

5 As used herein, "analyte" refers to any atom and/or  
6 molecule; including their complexes and fragment ions. In the  
7 case of biological molecules/macromolecules or "biopolymers",  
8 such analytes include but are not limited to: proteins,  
9 peptides, DNA, RNA, carbohydrates, steroids, and lipids. Note  
10 that most important biomolecules under investigation for their  
11 involvement in the structure or regulation of life processes  
12 are quite large (typically several thousand times larger than  
13 H<sub>2</sub>O.

14 As used herein, the term "molecular ions" refers to  
15 molecules in the charged or ionized state, typically by the  
16 addition or loss of one or more protons (H<sup>+</sup>).

17 As used herein, the term "molecular fragmentation" or  
18 "fragment ions" refers to breakdown products of analyte  
19 molecules caused, for example, during laser-induced desorption  
20 (especially in the absence of added matrix).

21 As used herein, the term "solid phase" refers to the  
22 condition of being in the solid state, for example, on the  
23 probe element surface.

1       As used herein, "gas" or "vapor phase" refers to molecules  
2       in the gaseous state (i.e., in vacuo for mass spectrometry).

3       As used herein, the term "analyte desorption/ionization"  
4       refers to the transition of analytes from the solid phase to  
5       the gas phase as ions. Note that the successful  
6       desorption/ionization of large, intact molecular ions by laser  
7       desorption is relatively recent (circa 1988)--the big  
8       breakthrough was the chance discovery of an appropriate matrix  
9       (nicotinic acid).

10       As used herein, the term "gas phase molecular ions" refers  
11       to those ions that enter into the gas phase. Note that large  
12       molecular mass ions such as proteins (typical mass=60,000 to  
13       70,000 times the mass of a single proton) are typically not  
14       volatile (i.e., they do not normally enter into the gas or  
15       vapor phase). However, in the procedure of the present  
16       invention, large molecular mass ions such as proteins do enter  
17       the gas or vapor phase.

18       As used herein in the case of MALDI, the term "matrix"  
19       refers to any one of several small, acidic, light absorbing  
20       chemicals (e.g., nicotinic or sinapinic acid) that is mixed in  
21       solution with the analyte in such a manner so that, upon drying  
22       on the probe element, the crystalline matrix-embedded analyte  
23       molecules are successfully desorbed (by laser irradiation) and

1 ionized from the solid phase (crystals) into the gaseous or  
2 vapor phase and accelerated as intact molecular ions. For the  
3 MALDI process to be successful, analyte is mixed with a freshly  
4 prepared solution of the chemical matrix (e.g., 10,000:1  
5 matrix:analyte) and placed on the inert probe element surface  
6 to air dry just before the mass spectrometric analysis. The  
7 large fold molar excess of matrix, present at concentrations  
8 near saturation, facilitates crystal formation and entrapment  
9 of analyte.

10 As used herein, "energy absorbing molecules (EAM)" refers  
11 to any one of several small, light absorbing chemicals that,  
12 when presented on the surface of a probe, facilitate the neat  
13 desorption of molecules from the solid phase (i.e., surface)  
14 into the gaseous or vapor phase for subsequent acceleration as  
15 intact molecular ions. The term EAM is preferred, especially in  
16 reference to SELDI. Note that analyte desorption by the SELDI  
17 process is defined as a surface-dependent process (i.e., neat  
18 analyte is placed on a surface composed of bound EAM). In  
19 contrast, MALDI is presently thought to facilitate analyte  
20 desorption by a volcanic eruption-type process that "throws"  
21 the entire surface into the gas phase. Furthermore, note that  
22 some EAM when used as free chemicals to embed analyte molecules  
23 as described for the MALDI process will not work (i.e., they do



1 not promote molecular desorption, thus they are not suitable  
2 matrix molecules).

3 As used herein, "probe element" or "sample presenting  
4 device" refers to an element having the following properties:  
5 it is inert (for example, typically stainless steel) and active  
6 (probe elements with surfaces enhanced to contain EAM and/or  
7 molecular capture devices).

8 As used herein, "MALDI" refers to Matrix-Assisted Laser  
9 Desorption/Ionization.

10 As used herein, "TOF" stands for Time-of-Flight.

11 As used herein, "MS" refers to Mass Spectrometry.

12 As used herein "MALDI-TOF MS" refers to Matrix-assisted  
13 laser desorption/ionization time-of-flight mass spectrometry.

14 As used herein, "ESI" is an abbreviation for Electrospray  
15 ionization.

16 As used herein, "chemical bonds" is used simply as an  
17 attempt to distinguish a rational, deliberate, and  
18 knowledgeable manipulation of known classes of chemical  
19 interactions from the poorly defined kind of general adherence  
20 observed when one chemical substance (e.g., matrix) is placed  
21 on another substance (e.g., an inert probe element surface).  
22 Types of defined chemical bonds include electrostatic or ionic  
23 (+/-) bonds (e.g., between a positively and negatively charged

1 groups on a protein surface), covalent bonds (very strong or  
2 "permanent" bonds resulting from true electron sharing),  
3 coordinate covalent bonds (e.g., between electron donor groups  
4 in proteins and transition metal ions such as copper or iron),  
5 and hydrophobic interactions (such as between two noncharged  
6 groups).

7 As used herein, "electron donor groups" refers to the case  
8 of biochemistry, where atoms in biomolecules (e.g, N, S, O)  
9 "donate" or share electrons with electron poor groups (e.g., Cu  
10 ions and other transition metal ions).

11 With the advent of mass spectroscopic methods such as  
12 MALDI and SELDI, researchers have begun to utilize a tool that  
13 holds the promise of uncovering countless biopolymers which  
14 result from translation, transcription and post-translational  
15 transcription of proteins from the entire genome.

16 Operating upon the principles of retentate chromatography,  
17 SELDI MS involves the adsorption of proteins, based upon their  
18 physico-chemical properties at a given pH and salt  
19 concentration, followed by selectively desorbing proteins from  
20 the surface by varying pH, salt, or organic solvent  
21 concentration. After selective desorption, the proteins  
22 retained on the SELDI surface, the "chip", can be analyzed  
23 using the CIPHERGEN protein detection system, or an equivalent

1     thereof. Retentate chromatography is limited, however, by the  
2     fact that if unfractionated body fluids, e.g. blood, blood  
3     products, urine, saliva, and the like, along with tissue  
4     samples, are applied to the adsorbent surfaces, the biopolymers  
5     present in the greatest abundance will compete for all the  
6     available binding sites and thereby prevent or preclude less  
7     abundant biopolymers from interacting with them, thereby  
8     reducing or eliminating the diversity of biopolymers which are  
9     readily ascertainable.

10         If a process could be devised for maximizing the diversity  
11     of biopolymers discernable from a sample, the ability of  
12     researchers to accurately determine the relevance of such  
13     biopolymers with relation to one or more disease states would  
14     be immeasurably enhanced.

1     SUMMARY OF THE INVENTION

2             The instant invention is characterized by the use of a  
3     combination of preparatory steps in conjunction with SELDI mass  
4     spectroscopy and time-of-flight detection procedures to  
5     maximize the diversity of biopolymers which are verifiable  
6     within a particular sample. The cohort of biopolymers verified  
7     within a sample is then viewed with reference to their ability  
8     to evidence at least one particular disease state; thereby  
9     enabling a diagnostician to gain the ability to characterize  
10    either the presence or absence of said at least one disease  
11    state relative to recognition of the presence and/or the  
12    absence of said biopolymer.

13            Although all manner of biomarkers related to all disease  
14    conditions are deemed to be within the purview of the instant  
15    invention and methodology, particular significance was given to  
16    those markers and diseases associated with the complement  
17    system and Syndrome X and diseases related thereto.

18            The complement system is an important part of non-clonal  
19    or innate immunity that collaborates with acquired immunity to  
20    destroy invading pathogens and to facilitate the clearance of  
21    immune complexes from the system. This system is the major  
22    effector of the humoral branch of the immune system, consisting  
23    of nearly 30 serum and membrane proteins. The proteins and

glycoproteins composing the complement system are synthesized largely by liver hepatocytes. Activation of the complement system involves a sequential enzyme cascade in which the proenzyme product of one step becomes the enzyme catalyst of the next step. Complement activation can occur via two pathways: the classical and the alternative. The classical pathway is commonly initiated by the formation of soluble antigen-antibody complexes or by the binding of antibody to antigen on a suitable target, such as a bacterial cell. The alternative pathway is generally initiated by various cell-surface constituents that are foreign to the host. Each complement component is designated by numerals (C1-C9), by letter symbols, or by trivial names. After a component is activated, the peptide fragments are denoted by small letters. The complement fragments interact with one another to form functional complexes. Ultimately, foreign cells are destroyed through the process of a membrane-attack complex mediated lysis.

The C4 component of the complement system is involved in the classical activation pathway. It is a glycoprotein containing three polypeptide chains ( $\alpha$ ,  $\beta$ , and  $\gamma$ ). C4 is a substrate of component C1s and is activated when C1s hydrolyzes a small fragment (C4a) from the amino terminus of the  $\alpha$  chain,

1 exposing a binding site on the larger fragment (C4b).

2 The native C3 component consists of two polypeptide  
3 chains,  $\alpha$  and  $\beta$ . As a serum protein, C3 is involved in the  
4 alternative pathway. Serum C3, which contains an unstable  
5 thioester bond, is subject to slow spontaneous hydrolysis into  
6 C3a and C3b. The C3f component is involved in the regulation  
7 required of the complement system which confines the reaction  
8 to designated targets. During the regulation process, C3b is  
9 cleaved into two parts: C3bi and C3f. C3bi is a membrane-bound  
10 intermediate wherein C3f is a free diffusible (soluble)  
11 component.

12 Complement components have been implicated in the  
13 pathogenesis of several disease conditions. C3 deficiencies  
14 have the most severe clinical manifestations, such as recurrent  
15 bacterial infections and immune-complex diseases, reflecting  
16 the central role of C3. The rapid profusion of C3f moieties  
17 and resultant "accidental" lysis of normal cells mediated  
18 thereby gives rise to a host of auto-immune reactions. The  
19 ability to understand and control these mechanisms, along with  
20 their attendant consequences, will enable practitioners to  
21 develop both diagnostic and therapeutic avenues by which to  
22 thwart these maladies.

23 In the course of defining a plurality of disease specific

1 marker sequences, special significance was given to markers  
2 which were evidentiary of a particular disease state or with  
3 conditions associated with Syndrome-X. Syndrome-X is a  
4 multifaceted syndrome, which occurs frequently in the general  
5 population. A large segment of the adult population of  
6 industrialized countries develops this metabolic syndrome,  
7 produced by genetic, hormonal and lifestyle factors such as  
8 obesity, physical inactivity and certain nutrient excesses.  
9 This disease is characterized by the clustering of insulin  
10 resistance and hyperinsulinemia, and is often associated with  
11 dyslipidemia (atherogenic plasma lipid profile), essential  
12 hypertension, abdominal (visceral) obesity, glucose intolerance  
13 or noninsulin-dependent diabetes mellitus and an increased risk  
14 of cardiovascular events. Abnormalities of blood coagulation  
15 (higher plasminogen activator inhibitor type I and fibrinogen  
16 levels), hyperuricemia and microalbuminuria have also been  
17 found in metabolic syndrome-X.

18 The instant inventors view the Syndrome X continuum in its  
19 cardiovascular light, while acknowledging its important  
20 metabolic component. The first stage of Syndrome X consists of  
21 insulin resistance, abnormal blood lipids (cholesterol and  
22 triglycerides), obesity, and high blood pressure  
23 (hypertension). Any one of these four first stage conditions

1 signals the start of Syndrome X.

2 Each first stage Syndrome X condition risks leading to  
3 another. For example, increased insulin production is  
4 associated with high blood fat levels, high blood pressure, and  
5 obesity. Furthermore, the effects of the first stage conditions  
6 are additive; an increase in the number of conditions causes an  
7 increase in the risk of developing more serious diseases on the  
8 Syndrome X continuum.

9 A patient who begins the Syndrome X continuum risks  
10 spiraling into a maze of increasingly deadly diseases. The next  
11 stages of the Syndrome X continuum lead to overt diabetes,  
12 kidney failure, and heart failure, with the possibility of  
13 stroke and heart attack at any time. Syndrome X is a dangerous  
14 continuum, and preventative medicine is the best defense.  
15 Diseases are currently most easily diagnosed in their later  
16 stages, but controlling them at a late stage is extremely  
17 difficult. Disease prevention is much more effective at an  
18 earlier stage.

19 Subsequent to the isolation of particular disease state  
20 marker sequences as taught by the instant invention, the  
21 promulgation of various forms of risk-assessment tests are  
22 contemplated which will allow physicians to identify  
23 asymptomatic patients before they suffer an irreversible event



1 such as diabetes, kidney failure, and heart failure, and enable  
2 effective disease management and preventative medicine.  
3 Additionally, the specific diagnostic tests which evolve from  
4 this methodology provide a tool for rapidly and accurately  
5 diagnosing acute Syndrome X events such as heart attack and  
6 stroke, and facilitate treatment.

7 Accordingly, it is an objective of the instant invention  
8 to define a disease specific marker sequence which is useful in  
9 evidencing and categorizing at least one particular disease  
10 state.

11 It is another objective of the instant invention to  
12 evaluate samples containing a plurality of biopolymers for the  
13 presence of disease specific marker sequences which evidence a  
14 link to at least one specific disease state.

15 It is a further objective of the instant invention to  
16 elucidate essentially all biopolymeric moieties contained  
17 therein, whereby particularly significant moieties may be  
18 identified.

19 It is a further objective of the instant invention provide  
20 at least one purified antibody which is specific to said  
21 disease specific marker sequence.

22 It is yet another objective of the instant invention to  
23 teach a monoclonal antibody which is specific to said disease

1 specific marker sequence.

2 It is a still further objective of the invention to teach  
3 polyclonal antibodies raised against said disease specific  
4 marker.

5 It is yet an additional objective of the instant invention  
6 to teach a diagnostic kit for determining the presence of said  
7 disease specific marker.

8 It is a still further objective of the instant invention  
9 to teach methods for characterizing disease state based upon  
10 the identification of said disease specific marker.

11 Other objectives and advantages of this invention will  
12 become apparent from the following description taken in  
13 conjunction with the accompanying drawings wherein are set  
14 forth, by way of illustration and example, certain embodiments  
15 of this invention. The drawings constitute a part of this  
16 specification and include exemplary embodiments of the present  
17 invention and illustrate various objects and features thereof.

18

19

20

1 BRIEF DESCRIPTION OF THE FIGURES

2 Figure 1 is a representation of derived data which  
3 characterizes a disease specific marker having a particular  
4 sequence useful in evidencing and categorizing at least one  
5 particular state;

6 Figure 2 is the characteristic profile derived via  
7 SELDI/TOF MS of the disease specific marker of Figure 1.  
8  
9

1     DETAILED DESCRIPTION OF THE INVENTION

2             Serum samples from individuals were analyzed using Surface  
3     Enhanced Laser Desorption Ionization (SELDI) using the  
4     Ciphergen PROTEINCHIP system. The chip surfaces included, but  
5     were not limited to IMAC-3-Ni, SAX2 surface chemistries, gold  
6     chips, and the like.

7             Preparatory to the conduction of the SELDI MS procedure,  
8     various preparatory steps were carried out in order to maximize  
9     the diversity of discernible moities educable from the sample.  
10    Utilizing a type of micro-chromatographic column called a C18-  
11    ZIPTIP available from the Millipore company, the following  
12    preparatory steps were conducted.

- 13            1. Dilute sera in sample buffer;
- 14            2. Aspirate and dispense ZIP TIP in 50% Acetonitrile;
- 15            3. Aspirate and dispense ZIP TIP in Equilibration;
- 16    solution;
- 17            4. Aspirate and Dispense in serum sample;
- 18            5. Aspirate and Dispense ZIP TIP in Wash solution;
- 19            6. Aspirate and Dispense ZIP TIP in Elution Solution.

20            Illustrative of the various buffering compositions useful  
21    in the present invention are:

22            Sample Buffers (various low pH's): Hydrochloric acid  
23    (HCl), Formic acid, Trifluoroacetic acid (TFA),

1        Equilibration Buffers (various low pH's): HCl, Formic  
2 acid, TFA;

3        Wash Buffers (various low pH's): HCl, Formic acid, TFA;

4        Elution Solutions (various low pH's and % Solvents): HCl,  
5 Formic acid, TFA;

6        Solvents: Ethanol, Methanol, Acetonitrile.

7        Spotting was then performed, for example upon a Gold Chip in  
8 the following manner:

9        1. spot 2 ul of sample onto each spot

10       2. let sample partially dry

11       3. spot 1 ul of matrix, and let air dry.

12       **HiQ Anion Exchange Mini Column Protocol**

13       1. Dilute sera in sample/running buffer;

14       2. Add HiQ resin to column and remove any air bubbles;

15       3. Add Ul water to aid in column packing;

16       4. Add sample/running buffer to equilibrate column;

17       5. Add diluted sera;

18       6. Collect all the flow through fraction in Eppendorf  
19 tubes until level is at resin;

20       7. Add sample/running buffer to wash column;

21       8. Add elution buffer and collect elution in Eppendorf  
22 tubes.

23       Illustrative of the various buffering compositions useful

1 in this technique are:

2 Sample/Running buffers: including but not limited to  
3 Bicine buffers of various molarities, pH's, NaCl content, Bis-  
4 Tris buffers of various molarities, pH's, NaCl content,  
5 Diethanolamine of various molarities, pH's, NaCl content,  
6 Diethylamine of various molarities, pH's, NaCl content,  
7 Imidazole of various molarities, pH's, NaCl content, Tricine of  
8 various molarities, pH's, NaCl content, Triethanolamine of  
9 various molarities, pH's, NaCl content, Tris of various  
10 molarities, pH's, NaCl content.

11 Elution Buffer: Acetic acid of various molarities, pH's,  
12 NaCl content, Citric acid of various molarities, pH's, NaCl  
13 content, HEPES of various molarities, pH's, NaCl content, MES  
14 of various molarities, pH's, NaCl content, MOPS of various  
15 molarities, pH's, NaCl content, PIPES of various molarities,  
16 pH's, NaCl content, Lactic acid of various molarities, pH's,  
17 NaCl content, Phosphate of various molarities, pH's, NaCl  
18 content, Tricine of various molarities, pH's, NaCl content.

19 **Chelating Sepharose Mini Column**

- 20 1. Dilute Sera in Sample/Running buffer;  
21 2. Add Chelating Sepharose slurry to column and allow  
22 column to pack;  
23 3. Add UF water to the column to aid in packing;

1        4.    Add Charging Buffer once water is at the level of the  
2 resin surface;

3        5.    Add UF water to wash through non bound metal ions  
4 once charge buffer washes through;

5        6.    Add running buffer to equilibrate column for sample  
6 loading;

7        7.    Add diluted serum sample;

8        8.    Add running buffer to wash unbound protein;

9        9.    Add elution buffer and collect elution fractions for  
10 analysis;

11       10.   Acidify each elution fraction.

12       Illustrative of the various buffering compositions useful  
13 in this technique are: Sample/Running buffers including but not  
14 limited to Sodium Phosphate buffers at various molarities and  
15 pH's;

16       Charging buffers including but not limited to Nickel  
17 Chloride, Nickel Sulphate, Copper II Chloride, Zinc Chloride or  
18 any suitable metal ion solution;

19       Elution Buffers including but not limited to Sodium  
20 phosphate buffers at various molarities and pH's containing  
21 various molarities of EDTA and/or Imidazole.

22

23

## HiS Cation Exchange Mini Column Protocol

1. Dilute sera in sample/running buffer;
2. Add HiS resin to column and remove any air bubbles;
3. Add Uf water to aid in column packing;
4. Add sample/running buffer to equilibrate column for sample loading;
5. Add diluted sera to column;
6. Collect all flow through fractions in Eppendorf tubes until level is at resin.
7. Add sample/running buffer to wash column.
8. Add elution buffer and collect elution in Eppendorf tubes.

Illustrative of the various buffering compositions useful in this technique are:

Sample/Running buffers: including but not limited to Bicine buffers of various molarities, pH's, NaCl content, Bis-Tris buffers of various molarities, pH's, NaCl content, Diethanolamine of various molarities, pH's, NaCl content, Diethylamine of various molarities, pH's, NaCl content, Imidazole of various molarities, pH's, NaCl content, Tricine of various molarities, pH's, NaCl content, Triethanolamine of various molarities, pH's, NaCl content, Tris of various molarities, pH's, NaCl content.



1 Elution Buffer: Acetic acid of various molarities,  
2 pH's, NaCl content, Citric acid of various molarities, pH's,  
3 NaCl content, HEPES of various molarities, pH's, NaCl  
4 content, MES of various molarities, pH's, NaCl content, MOPS  
5 of various molarities, pH's, NaCl content, PIPES of various  
6 molarities, pH's, NaCl content, Lactic acid of various  
7 molarities, pH's, NaCl content, Phosphate of various  
8 molarities, pH's, NaCl content, Tricine of various  
9 molarities, pH's, NaCl content.

10 The procedure for profiling serum samples is described  
11 below:

12 Following the preparatory steps illustrated above,  
13 various methods for use of the PROTEINCHIP arrays, available  
14 for purchase from CIPHERGEN Biosystems (Palo Alto, CA), may  
15 be practiced. Illustrative of one such method is as follows.

16 The first step involved treatment of each spot with 20  
17 ml of a solution of 0.5 M EDTA for 5 minutes at room  
18 temperature in order to remove any contaminating divalent  
19 metal ions from the surface. This was followed by rinsing  
20 under a stream of ultra-filtered, deionized water to remove  
21 the EDTA. The rinsed surfaces were treated with 20 ml of 100  
22 mM Nickel sulfate solution for 5 minutes at room temperature

1 after which the surface was rinsed under a stream of ultra-  
2 filtered, deionized water and allowed to air dry.

3 Serum samples (2 ml) were applied to each spot (now  
4 "charged" with the metal-Nickel) and the PROTEINCHIP was  
5 returned to the plastic container in which it was supplied.  
6 A piece of moist KIMWIPE was placed at the bottom of the  
7 container to generate a humid atmosphere. The cap on the  
8 plastic tube was replaced and the chip allowed to incubate at  
9 room temperature for one hour. At the end of the incubation  
10 period, the chip was removed from the humid container and  
11 washed under a stream of ultra-filtered, deionized water and  
12 allowed to air dry. The chip surfaces (spots) were now  
13 treated with an energy-absorbing molecule that helps in the  
14 ionization of the proteins adhering to the spots for analysis  
15 by Mass Spectrometry. The energy-absorbing molecule in this  
16 case was sinapinic acid and a saturated solution prepared in  
17 50% acetonitrile and 0.05% TFA was applied (1 ml) to each  
18 spot. The solution was allowed to air dry and the chip  
19 analyzed immediately using MS (SELDI).

20 Serum samples from patients suffering from a variety of  
21 disease states were analyzed using one or more protein chip  
22 surfaces, e.g. a gold chip or an IMAC nickel chip surface as  
23 described above and the profiles were analyzed to discern

1 notable sequences which were deemed in some way evidentiary  
2 of at least one disease state.

3 In order to purify the disease specific marker and  
4 further characterize the sequence thereof, additional  
5 processing was performed.

6 For example, Serum (20 ml) was (diluted 5-fold with  
7 phosphate buffered saline) concentrated by centrifugation  
8 through a YM3 MICROCON spin filter (Amicon) for 20 min at  
9 10,000 RPM at 4°C in a Beckman MICROCENTRIFuge R model bench  
10 top centrifuge. The filtrate was discarded and the retained  
11 solution, which contained the two peptides of interest, was  
12 analyzed further by tandem mass spectrometry to deduce their  
13 amino acid sequences. Tandem mass spectrometry was performed  
14 at the University of Manitoba's (Winnipeg, Manitoba, Canada)  
15 mass spectrometry laboratory using the procedures that are  
16 well known to practitioners of the art.

17 As a result of these procedures, the disease specific  
18 marker identified by the sequence SSKITHRIHWESASLLR was  
19 found. This marker is characterized as a C3f fragment from  
20 the complement system having a molecular weight of about 1998  
21 daltons. The characteristic profile of the marker is set  
22 forth in Figure 2. As easily deduced from the data set forth

b1  
c2

1 ~~in Figure 1, this marker is indicative of an individual~~  
2 ~~suffering from Type II diabetes.~~

3 In accordance with various stated objectives of the  
4 invention, the skilled artisan, in possession of the specific  
5 disease specific marker as instantly disclosed, would readily  
6 carry out known techniques in order to raise purified  
7 biochemical materials, e.g. monoclonal and/or polyclonal  
8 antibodies, which are useful in the production of methods and  
9 devices useful as point-of-care rapid assay diagnostic or  
10 risk assessment devices as are known in the art.

11 The specific disease markers which are analyzed  
12 according to the method of the invention are released into  
13 the circulation and may be present in the blood or in any  
14 blood product, for example plasma, serum, cytolyzed blood,  
15 e.g. by treatment with hypotonic buffer or detergents and  
16 dilutions and preparations thereof, and other body fluids,  
17 e.g. CSF, saliva, urine, lymph, and the like. The presence  
18 of each marker is determined using antibodies specific for  
19 each of the markers and detecting specific binding of each  
20 antibody to its respective marker. Any suitable direct or  
21 indirect assay method may be used to determine the level of  
22 each of the specific markers measured according to the  
23 invention. The assays may be competitive assays, sandwich

1 assays, and the label may be selected from the group of well-  
2 known labels such as radioimmunoassay, fluorescent or  
3 chemiluminescence immunoassay, or immunoPCR technology.  
4 Extensive discussion of the known immunoassay techniques is  
5 not required here since these are known to those of skilled  
6 in the art. See Takahashi et al. (Clin Chem 1999;45(8):1307)  
7 for S100B assay.

8 A monoclonal antibody specific against the disease  
9 marker sequence isolated by the present invention may be  
10 produced, for example, by the polyethylene glycol (PEG)  
11 mediated cell fusion method, in a manner well-known in the  
12 art.

13 Traditionally, monoclonal antibodies have been made  
14 according to fundamental principles laid down by Kohler and  
15 Milstein. Mice are immunized with antigens, with or without,  
16 adjuvants. The splenocytes are harvested from the spleen for  
17 fusion with immortalized hybridoma partners. These are  
18 seeded into microtitre plates where they can secrete  
19 antibodies into the supernatant that is used for cell  
20 culture. To select from the hybridomas that have been plated  
21 for the ones that produce antibodies of interest the  
22 hybridoma supernatants are usually tested for antibody  
23 binding to antigens in an ELISA (enzyme linked immunosorbent

1 assay) assay. The idea is that the wells that contain the  
2 hybridoma of interest will contain antibodies that will bind  
3 most avidly to the test antigen, usually the immunizing  
4 antigen. These wells are then subcloned in limiting dilution  
5 fashion to produce monoclonal hybridomas. The selection for  
6 the clones of interest is repeated using an ELISA assay to  
7 test for antibody binding. Therefore, the principle that has  
8 been propagated is that in the production of monoclonal  
9 antibodies the hybridomas that produce the most avidly  
10 binding antibodies are the ones that are selected from among  
11 all the hybridomas that were initially produced. That is to  
12 say, the preferred antibody is the one with highest affinity  
13 for the antigen of interest.

14 There have been many modifications of this procedure  
15 such as using whole cells for immunization. In this method,  
16 instead of using purified antigens, entire cells are used for  
17 immunization. Another modification is the use of cellular  
18 ELISA for screening. In this method instead of using  
19 purified antigens as the target in the ELISA, fixed cells are  
20 used. In addition to ELISA tests, complement mediated  
21 cytotoxicity assays have also been used in the screening  
22 process. However, antibody-binding assays were used in  
23 conjunction with cytotoxicity tests. Thus, despite many

1 modifications, the process of producing monoclonal antibodies  
2 relies on antibody binding to the test antigen as an  
3 endpoint.

4 The purified monoclonal antibody is utilized for  
5 immunochemical studies.

6 Polyclonal antibody production and purification  
7 utilizing one or more animal hosts in a manner well-known in  
8 the art can be performed by a skilled artisan.

9 Another objective of the present invention is to provide  
10 reagents for use in diagnostic assays for the detection of  
11 the particularly isolated disease specific marker sequences  
12 of the present invention.

13 In one mode of this embodiment, the marker sequences of  
14 the present invention may be used as antigens in immunoassays  
15 for the detection of those individuals suffering from the  
16 disease known to be evidenced by said marker sequence. Such  
17 assays may include but are not limited to: radioimmunoassay,  
18 enzyme-linked immunosorbent assay (ELISA), "sandwich" assays,  
19 precipitin reactions, gel diffusion immunodiffusion assay,  
20 agglutination assay, fluorescent immunoassays, protein A or G  
21 immunoassays and immunoelectrophoresis assays.

22 According to the present invention, monoclonal or  
23 polyclonal antibodies produced against the disease specific

1 marker sequence of the instant invention are useful in an  
2 immunoassay on samples of blood or blood products such as  
3 serum, plasma or the like, spinal fluid or other body fluid,  
4 e.g. saliva, urine, lymph, and the like, to diagnose patients  
5 with the characteristic disease state linked to said marker  
6 sequence. The antibodies can be used in any type of  
7 immunoassay. This includes both the two-site sandwich assay  
8 and the single site immunoassay of the non-competitive type,  
9 as well as in traditional competitive binding assays.

10 Particularly preferred, for ease and simplicity of  
11 detection, and its quantitative nature, is the sandwich or  
12 double antibody assay of which a number of variations exist,  
13 all of which are contemplated by the present invention. For  
14 example, in a typical sandwich assay, unlabeled antibody is  
15 immobilized on a solid phase, e.g. microtiter plate, and the  
16 sample to be tested is added. After a certain period of  
17 incubation to allow formation of an antibody-antigen complex,  
18 a second antibody, labeled with a reporter molecule capable  
19 of inducing a detectable signal, is added and incubation is  
20 continued to allow sufficient time for binding with the  
21 antigen at a different site, resulting with a formation of a  
22 complex of antibody-antigen-labeled antibody. The presence  
23 of the antigen is determined by observation of a signal which



1 may be quantitated by comparison with control samples  
2 containing known amounts of antigen.

3 All patents and publications mentioned in this  
4 specification are indicative of the levels of those skilled  
5 in the art to which the invention pertains. All patents and  
6 publications are herein incorporated by reference to the same  
7 extent as if each individual publication was specifically and  
8 individually indicated to be incorporated by reference.

9 It is to be understood that while a certain form of the  
10 invention is illustrated, it is not to be limited to the  
11 specific form or arrangement herein described and shown. It  
12 will be apparent to those skilled in the art that various  
13 changes may be made without departing from the scope of the  
14 invention and the invention is not to be considered limited  
15 to what is shown and described in the specification and  
16 drawings/figures.

17 One skilled in the art will readily appreciate that the  
18 present invention is well adapted to carry out the objectives  
19 and obtain the ends and advantages mentioned, as well as  
20 those inherent therein. The oligonucleotides, peptides,  
21 polypeptides, biologically related compounds, methods,  
22 procedures and techniques described herein are presently  
23 representative of the preferred embodiments, are intended to

1 be exemplary and are not intended as limitations on the  
2 scope. Changes therein and other uses will occur to those  
3 skilled in the art which are encompassed within the spirit of  
4 the invention and are defined by the scope of the appended  
5 claims. Although the invention has been described in  
6 connection with specific preferred embodiments, it should be  
7 understood that the invention as claimed should not be unduly  
8 limited to such specific embodiments. Indeed, various  
9 modifications of the described modes for carrying out the  
10 invention which are obvious to those skilled in the art are  
11 intended to be within the scope of the following claims.